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(71) Applicant *(for all designated States except US)*: LUDWIG INSTITUTE FOR CANCER RESEARCH [US/US]; 1345 Avenue of the Americas, New York, NY 10105 (US).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)* : ICHIJO, Hidenori [JP/SE]; MIYAZONO, Kohei [JP/SE]; RÖNNSTRAND, Lars [SE/SE]; HELLMAN, Ulf [SE/SE]; WERNSTEDT, Christer [SE/SE]; HELDIN, Carl-Henrik [SE/SE]; Biomedical Center, Husargatan 3, S-751 01 Uppsala (SE).

(74) Agent: HANSON, Norman, D.; Felfe & Lynch, 805 Third Avenue, New York, NY 10022 (US).

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(54) Title: SUBSTANTIALLY PURE RECEPTOR LIKE TGF-β1 BINDING MOLECULES AND USES THEREOF

(57) Abstract

The invention relates to a family of substantially pure, receptor like TGF-β1 binding glycoproteins. These molecules are characterized by molecular masses of 160 kd, 70-80 kd, and 30-40 kd as determined by SDS-PAGE, and the ability to bind the TGF-β1 molecule. This family of molecules is useful in identifying and/or quantifying TGF-β1 in a sample, as well as inhibiting its effect on cells. Also described are nucleic acid sequences which code for the protein monomer making up the molecules.

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SUBSTANTIALLY PURE RECEPTOR LIKE TGF-ß1 BINDING MOLECULES AND USES THEREOF

RELATED APPLICATION

This application is a continuation in part of U.S. patent application Serial Number 717,316, filed on June 18, 1991.

FIELD OF THE INVENTION

This invention relates to protein biochemistry. More particularly, it relates to molecules which bind to the substance known as transforming growth factor-ß1 ("TGF-ß1" hereafter). The invention also relates to nucleic acid sequences coding for the molecule, and uses thereof.

BACKGROUND AND PRIOR ART

A family of molecules is referred to as the "TGF-ßs". These are 25 kd dimeric proteins which have multi-functional effects on growth and differentiation of cells, both _in vitro_ and _in vivo_. See Roberts et al. in Peptide Growth Factors And Their Receptors I (Sporn et al., eds., pp 419-472; Springer-Verlag, Berlin, 1990); Moses et al., Cell 63: 245-247 (1990); Massagué, Ann. Rev. Cell. Biol. 6: 597-641 (1990). The family contains at least three different, structurally related members, identified as "ß1, ß2 and ß3". Many other proteins are more distantly related, including bone morphogenic proteins, Müllerian inhibitory substance, activins, inhibins, and so forth.

Originally, the TGF-ß family of proteins was identified as being involved in increasing anchorage independent growth of normal rat kidney cells; however, the proteins are also recognized as a potent growth inhibitor for diverse cell types, including hematopoietic cells, lymphocytes, epithelial and endothelial cells (Ohta et al., Nature 329: 539-541 (1987); Kehri et al., J. Immunol 137: 3855-3860 (1986); Moses et al., in Cancer Cells 3 (Feramisco et al., ed; Cold Spring Harbor, New York, 1985);

pg. 65-71; Baird et al.. Biochem. Biophys. Res. Commun 138: 476-482 (1986); Frater-Schröder et al., Biochem. Biophys. Res. Commun. 137: 295-302 (1986); Heimark et al., Science 233: 1078-1080 (1986)). The molecules have a dramatic effect on accumulation of extracellular matrix proteins (Massagué, supra), and have been implicated in pathogenesis glomerulonephritis (Border et al., Nature 346: 371-374 (1990)); liver cirrhosis (Castilla et al., N. Eng. J. Med. 324: 933-940 (1990)); and pulmonary fibrosis (Khalil et al., in Clinical Application of TGF-ß1 (Bock et al., ed. Ciba Foundation Symposium 157, John Willy & Sons, 1991, pg. 194-211).

The TGF-ß family interacts with other proteins on several levels. One of these is mediation of binding via cell surface receptors. The art recognizes three distinct high affinity receptors for TGF-ßs, referred to as types I, II and III. The first two of these have molecular masses of 53 and 70-85 kd, respectively, while the third is denoted "betaglycan" because of its proteoglycan like structure, and is further characterized by a molecular mass of 200-400 kd. Massagué et al., in Transforming Growth Factor-ßs: Chemistry, Biology and Therapeutics (Piez et al., eds., Ann. N.Y. Acad. Sci. 593, 1990), pg. 59-72; Segarini et al., in Clinical Applications of TGF-ß (Bock et al., eds. Ciba Foundation Symposium 157, John Wiley & Sons, 1991, pg 29-50). The betaglycan molecule is a membrane proteoglycan, having a 100-140 kd core protein with unknown functional importance, while type I and II receptors appear to be involved in transduction of TGF-ß cellular effect. Segarini et al., J. Biol. Chem. 263: 8366-8370 (1988); Cheifetz et al., J. Biol. Chem. 263: 16884-16991 (1988); Massagué et al., supra. Some cell lines express only type I receptors and are inhibited by TGF-ß1. These include hematopoietic progenitor cell lines (Ohta et al., supra) and squamous cancer cell lines (Ichiyo et al., Exp. Cell Res. 187: 263-269 (1990)). Mutant cell lines of mink epithelial cells have been shown to have lost or to have

anomalous expression of type I and/or type II receptors (Boyd et al., J. Biol. Chem. 264: 2272-2278 (1989); Laiho et al., J. Biol. Chem. 265: 18518-18524 (1990)).

Additional binding molecules for TGF-ß having molecular masses of 60 kd, 85-320 kd, and 400 kd have been reported in pituitary tumor cell lines, rat glomeruli, and bovine liver cells, respectively, as reported by Cheifetz et al., J. Biol. Chem. 263: 17225-17228 (1988); Mackay et al., J. Biol. Chem. 265: 9351-9356 (1990); O'Grady et al., J. Biol. Chem. 266: 8583-8589 (1991).

On another level, the precursors of TGF-ß, especially TGF-ß1, interact with protein molecules known as the latent TGF-binding protein or "LTBP". The interaction yields a high molecular weight, inactive complex which is secreted from the cell. This is sometimes referred to as the latent TGF-ß1 complex. See Miyazono et al., J. Biol. Chem. 263: 6407-6415 (1988); Pircher et al., Biochem. Biophys. Res. Commun. 136: 30-37 (1984); Wakefield et al., J. Cell Biol. 105: 965-975 (1987). The inactive or latent complexes contain a non-covalent association of TGF-ß1, a disulphide bonded complex of a dimer of N-terminal peptide of TGF-ß1 precursor and as third component, the LTBP. This third component occurs as a molecule with a molecular mass which may range from 125-190 kds. Experiments have shown that the binding proteins do not inactivate TGF-ß1.

The molecules discussed <u>supra</u> are sometimes referred to as "binding proteins", because they do, in fact bind to the TGF-ß1 precursor. A fundamental difference between these molecules and the molecules of the invention is that while the prior art molecules may be referred to as "synthesis" binders, it is more appropriate to describe the invention as involving "effector" binders. The synthesis binders are involved in the "packaging" of TGF-ß1 in the cell, such that it is released for subsequent activities. When bound to the prior art molecules, TGFs are essentially inert. In contrast, the protein containing molecules of the invention may be seen as "effectors" in that TGF-ß1

binds directly to these, so as to effect a response thereby. This distinction should be kept in mind in connection with this application.

It is an object of the invention to describe these substantially pure, receptor like TGF-ß1 binding protein containing molecules, which are characterized by molecular masses of 160 kd, 70-80 kd, and 35-40 kd as determined by SDS-PAGE, as well as their uses in various processes. The ranges are due to the behavior of the species under reducing and non-reducing conditions, as will be seen _infra_. It is also due to the nature of the molecule's structure, which is as a monomer, dimer or trimer based upon a single peptide. The implication of this structure are discussed _infra_.

The objects of the invention discussed _supra_ as well as others will be seen from the disclosure which follows.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 provides a schematic outline of the purification protocol used to isolate the substantially pure receptor like transforming growth factor ß1 binding proteins of the invention.

Figure 2 shows a protein profile of a representative FPLC run for the binding proteins of the invention.

Figure 3 shows SDS gel runs for various fractions prepared according to the invention, following affinity cross linking with ^{125}I-TGF-ß1.

Figure 4 depicts "in-gel" ligand binding of fractions following FPLC Mono-Q chromatography.

Figure 5 shows SDS-gel electrophoretic analysis of fractions obtained following sepharose chromatography using TGF-ß1.

Figure 6 presents analysis of the pH 3.5 elution fraction of TGF-ß1 Sepharose chromatography, following affinity labelling using ^{125}I-TGF-ß1.

Figure 7 presents an analysis of different TGF-ß1 Sepharose chromatography fractions using "in-gel" ligand binding.

Figure 8 is a protein profile of a chromatogram of the pH 3.5 elution fraction, following acetone precipitation concentration.

Figure 9 is an SDS-gel analysis of various fractions obtained from Superose 12 chromatography.

Figure 10 shows analysis of the pure 40 kd component of the analysis, using ^{125}I-TGF-ß1 affinity cross linking experiments.

Figure 11 shows in gel binding of the 40 kd receptor like binding protein.

DETAILED DESCRIPTION OF PREFERRED EMBODIMENTS

Example 1

A protocol originally described by Rönnstrand et al., J. Biol. Chem. 262: 2929-2932 (1987) for purifying PDGF receptor was followed. Briefly, porcine uterus tissue was used as starting material for preparation of membranes. Differential centrifugation following Rönnstrand, supra, was used to obtain the membranes. The membrane proteins were then solubilized in Triton X-100^{R}, and subjected to chromatography on wheat germ agglutinin Sepharose and fast protein liquid chromatography Mono-Q columns. First, the proteins were purified on the wheat germ agglutinin column, and the purified material was then applied to an FPLC Mono Q column, using increasing concentrations of NaCl. Twenty-

six fractions were taken from the column. Of these, fractions 16-20 were pooled and used to purify PDGF receptor; the other fractions were stored at -20°C and used as starting material to purify the receptor like binding proteins of the invention. Figure 2 shows the concentration of NaCl used for each fraction, via the dotted line. Fractions 16-20 were used for PDGF purification.

Example 2

To remove the receptor like binding proteins from the fractions obtained following Example 1, a TGF-ß1 Sepharose column was prepared. This was accomplished using recombinant TGF-ß1 purified from conditioned medium of CHO cells, transfected with human TGF-ß1 cDNA. One mg of TGF-ß1 was coupled to 0.5 g of cyanogen bromide activated Sepharose 4B, to yield about 0.67 mg of TGF-ß1/ml of gel.

Following preparation of the column, fractions 1-15 and 21-26 from example 1 were thawed, pooled, and dialyzed against binding buffer (0.2% Triton X-100^R, 125 mM NaCl, 5 mM KCl, 5mM $MgSO_4$, 1.2 mM $CaCl_2$, 20 mM HEPES, pH 7.4). Twenty-five ml amounts of dialyzed sample were then mixed with 2.5 ml of the previously prepared Sepharose beads, and the resulting suspension was incubated overnight at 4°C, with gentle shaking. The beads were then collected in a column which was then washed with 25 ml of binding buffer, and then 25 ml of binding buffer with 500 mM NaCl. Bound molecules were then eluted with 5 ml of a solution of 0.2% Triton X-100^R, 500 mM NaCl, in 100 mM sodium acetate buffer, pH 5.5, followed by 5 ml of 0.2% Triton X-100^R, 500 mM NaCl, and 100 mM acetic acid at pH 3.5, to yield what will be referred to as the "pH 3.5 eluate" hereafter.

Example 3

The pH 3.5 eluate fractions from four to six chromatography runs, as discussed supra, were pooled and

mixed with four volumes of acetone. The protein portion was precipitated at -20°C for 60 minutes, followed by centrifugation at 17,000xg at 4°C for 20 minutes. The resulting protein pellets were dried, resuspended in 500 ul of 70% formic acid, followed by application to an FPLC Superose 12 column which had been pre-equilibrated and eluted with 70% formic acid at a flow rate of 0.5 ml/min. Fractions (250 ul) were collected, and aliquots of individual fractions were lyophilized and subjected to further analysis.

__Example 4__

Fractions obtained following example 2 were examined for ^{125}I-TGF-ß1 binding, using affinity cross linking and "in gel" binding procedures.

To do this, 50 ul portions of the individual Mono-Q fractions were incubated for three hours at 4°C in the presence of 1 nM of recombinant TGF-ß1 labeled with ^{125}I following Frolik et al., J. Biol. Chem. 259: 10995-11000 (1984), to yield a product with $5x10^6$ cpm/ml. The incubation took place with fractions that either had been dialyzed against the binding buffer described __supra__, or dissolved in it.

The affinity labeled proteins were then cross-linked using 0.14 mM of disuccinimidyl suberate ("DSS") for 15 minutes at 4°C. The cross-linking reaction was quenched by adding SDS-electrophoresis sample buffer containing 80 mM Tris. In this and in following examples, the samples were then heated at 95°C for three minutes in SDS-sample buffer which did or did not contain 10 mM dithiothreitol (DTT). The samples were then applied to 5-15% SDS-polyacrylamide gels following Blobel et al., J. Cell Biol. 67: 835-851 (1975) for electrophoresis, under either reducing or non-reducing conditions. Gels were then fixed in 25% methanol, 7.5% acetic acid, and dried and subjected to 12-days of

autoradiography using Fuji X-ray film. The results of the autoradiography are shown in Figure 3.

Example 5

Experiments were also carried out to study "in gel" binding using radiolabeled TGF-ß1. To do this, the method described by Murphy et al., Anal. Biochem. 187: 197-201 (1990) for ^{125}I heparin, was used with some minor modifications. To summarize, 250 ul of individual fractions were lyophilized and subjected to non-reducing SDS gel electrophoresis. Following SDS-gel electrophoresis, gels were fixed for 30 minutes in 40% methanol, 7% acetic acid, and rinsed several times with distilled water. The gels were then incubated overnight with 10% ethanol, 10 mM Tris-HCl, pH 7.5 at 4°C with gentle shaking, followed by washing for one hour with the same buffer. The gels were then incubated for 30 minutes with binding buffer containing bovine serum albumin (BSA) at 2 mg/ml. Gels were then transferred to plastic bags with 10 ml binding buffer containing $1x10^6$ cpm of ^{125}I-TGF-ß1 and 2 mg/ml of BSA. These bags were sealed and shaken overnight at 4°C. Excess ^{125}I-TGF-ß1 was removed, and the gels were then washed with 500 ml of binding buffer for 30 minutes, followed by two washes with 500 mM binding buffer containing 400 mM NaCl. Each wash was for 30 minutes. Following this, gels were dried and subjected to autoradiography as per example 4, but for three days. Figure 4 shows these results.

In both of examples 4 and 5, ^{14}C labeled molecular weight markers were used as follows: myosin (200 kd), phosphorylase B (92.5 kd) bovine serum albumin (69 kd), ovalbumin (46 kd), carbonic anhydrase (30 kd), lysozyme (14.3 kd).

The results from example 4 showed complexes of apparent molecular masses of 210,000; 170,000; and 145,000 kd from most of the fractions, as well as complexes with

molecular masses of 80,000 and 65,000 in fractions 2-10, plus one of 53,000 in fractions 10-20. Example 5 (Fig. 4) results showed several binding components in the 90-200 kd range in fractions 5-26, plus a prominent band of 36 kd in fractions 11-26.

Example 6

The results obtained in Example 4 gave a pattern which was similar to that observed by Seganini et al., J. Biol. Chem. 263: 8366-8370 (1988), and Cheifetz et al., J. Biol. Chem. 263: 16884-16991 (1988), for betaglycan affinity labeled with ^{125}I-TGF-ß1. To determine whether either of the 210 kd or 170 kd components represented betaglycan, samples were again cross linked with ^{125}I-TGF-ß1, and digested with heparinase and chondroitinase. The references cited to supra had shown that the proteoglycan betaglycan shifts to 100-140 kd following such treatments. The 210 and 170 kd complexes tested herein showed no such movement, strongly suggesting that they did not represent betaglycan.

Example 7

Once it had been shown that there was binding activity for the proteins of examples 1-6, further purification steps were carried out.

Again referring to the fraction obtained with the Mono-Q column, all but fractions 16-20 were combined and dialyzed against dialysis buffer as described supra. Again, following the protocols described supra the dialyzed material was subjected to affinity chromatography using immobilized TGF-ß1. Following this, the column was washed with binding buffer, described supra, then with binding buffer at higher ionic strength, i.e., 0.5 M NaCl. Following this, two elutions were carried out, first with an elution buffer at pH 5.5, and than at pH 3.5 Each

fraction was analyzed on SDS-PAGE, using silver staining in the absence and presence of reducing agent.

The results from these different fractionations are shown in Figure 5, where "FT" refers to the flow through material, "W1" to the low ionic strength wash, "W2" to the high ionic strength wash, "E1" to the elution at pH 5.5, and E2 to the elution at pH 3.5. Very little protein eluted at pH 5.5, while at pH 3.5 and under non reducing conditions, materials eluted which showed apparent molecular masses of 160, 72, 46 and 36 kilodaltons. When this fraction was tested under reducing conditions, species of apparent molecular masses of 160, 80, 50 and 40 kilodaltons were observed. This suggests that four separate species were present, having molecular weights of 160 kd, and ranging from 70-80 kd, 45-50 kd, and 35-40 kd.

Example 8

The pH 3.5 eluate clearly contained the material of interest, and was subjected to further analysis. An aliquot of the fraction was lyophilized, redissolved in binding buffer, and incubated with 1 nM ^{125}I-TGF-ß1, prepared as described supra, either without or with an excess amount of unlabeled TGF-ß1 (400 nM). Again, following the protocols set forth supra, these materials were cross linked with DSS and analyzed via SDS gel electrophoresis under reducing conditions. Radiographic data from these experiments are presented in Figure 6, and show complexes with apparent molecular weights of 170 and 53 kd. These complexes were associated with ^{125}I-TGF-ß1. This radiolabelled molecule has a molecular weight of 12.5 kd under reduced conditions, so it would appear that the binding materials are the 160 and 40 kd species of Example 7. Components of molecular mass 70-90 kd and 25 kd were also found, but there was no observed displacement even when 400 fold cold molar excesses of unlabeled TGF-ß1 were used. Also, these bands were found in control lanes where

samples were not present, suggesting free ^{125}I-TGF-ß1 and the labeled molecule non-specifically cross linked to BSA.

Example 9

The pH 3.5 fraction was then used for "in gel" ligand binding, as were some of the other fractions. Specifically, 70 ul of FT, E1 and E2 fractions were subjected to the same protocol for in gel ligand binding as described previously. Figure 7 shows these results. Two of the three components recognized were identical to components found using the affinity labeling experiment of Example 8. A third, an 80 kd band, may have been hidden in the diffuse 70-90 kd band shown in Figure 6 and discussed in Example 8. A fourth component, a 50 kd band, was the material present in lowest quantity in the pH 3.5 fraction.

Example 10

The foregoing examples showed that there were several receptor like binding proteins present. In order to separate these, a size separation method was used. Specifically, pH 3.5 eluates from four to six TGF-ß1 Sepharose chromatographies were pooled and subjected to acetone precipitation, as per Example 2, supra. Precipitates were dried, redissolved in 70% formic acid, and applied to an FPLC Superose 12 column eluted in 70% formic acid.

The protein profile of this chromatography is shown in Figure 8, where three major protein peaks were found at fractions 28-31, 32-34, and 44-48. A shoulder was found at fractions 36-40.

Individual fractions 31-48 were then lyophilized and analyzed in 10 ul aliquots, using SDS-PAGE under non reducing conditions followed by silver staining. These results are shown in Figure 9. They show that the 160 kd component eluted in a broad peak through fractions 32-42, while a 72 kd component eluted in fractions 37-40, and the 36 kd component in fractions 44-47. This last material was

apparently homogeneous, and further analysis under reducing conditions showed a 40 kd fraction. These results indicate that this material is a single chain polypeptide, probably containing intra-chain disulphide bonds. The absorbance values at 280 nm in Figure 8 suggest that about 12 ug of this 40 kd molecule can be purified from 10 kg of tissue.

Example 11

The 40 kd molecule was analyzed to test its binding to TGF-ß1, using the affinity cross-linking protocols set forth supra. Figure 10 shows that this experiment yielded a 62 kd complex under non-reducing conditions, and a 53 kd complex when 10 mM of DTT are present. If the molecular mass of TGF-ß1 under non-reducing and reducing conditions are subtracted (25 kd, 12.5 kd), the resulting figure is 40 kd.

Similarly, when "in-gel" binding under non-reducing conditions was carried out, a labeled band is found at 36 kd, as shown in Figure 11. This is the expected value, and the results prove that the substantially pure receptor like binding protein for TGF-ß1 having a molecular mass of 36-40 kd does bind the molecule when in homogeneous form.

Example 12

The initial purification work, as per Example 1, used a wheat germ agglutinin column, so it cannot be ruled out that the materials of interest are glycoproteins. To that end, the molecules of the invention are described as "protein containing" because they definitely contain a protein component, and may be glycoproteins. Analysis of the homogeneous 40 kd component using endoglycosidase F, using SDS PAGE, silver staining and reducing conditions yielded a 35 kd product, so the molecules may be glycoproteins.

Example 13

Tryptic digestion of the 40 kd material has revealed some amino acid data. The following sequences have been identified:

(I) V(D)LV(D)FEGNHQFA

(II) VVGLEGSDKLSILR

(III) VFGSQLGE

where P* is hydroxyproline, and a bracketed amino acid means the determination is tentative. Sequence III has been used to prepare antiserum which specifically binds to all three glycoproteins.

Example 14

Additional experiments with the proteins described supra resulted in the generation of additional tryptic fragments. These included the following:

~~(1) YLGGSHGSFA~~

(2) VVGLEGSDKLSILR

(3) CP*GLP*GAAGP

(4) DWAAY

An additional peptide was derived from lysine peptidase fragment digestion of a mixture of the 3 glycoproteins discussed supra.

RGFGSQLGEFWLGNDHIHALTAQGTNELXVDLVFEGNHQFA.

Example 15

Experiments were carried out to isolate a cDNA sequence coding for the protein of interest. In order to prepare a specific probe, degenerate oligomers were used based upon the peptide sequences presented in Example 13 and 14, in a polymerase chain reaction ("PCR") using mRNA derived from porcine uterus. Specifically the sequences

QLGEFW

and

FEGNHQF

were used to prepare sense and antisense degenerate oligomers:

```
5' CAA  CTN  GGN  GAA  TTT  TGG-3'
     G   T         G    C
                                (sense)

5' AAA  TTG  ATG  ATT  NCC  TTC  AAA-3'
    G    C    G    G         C    G
                                (antisense).
```

These degenerate oligomers were used in the polymerase chain reaction on the mRNA, and this led to the amplification of an approximately 100 base pair fragment. The 100 base pair fragment was subcloned into bluescript, and was then sequenced.

__Example 16__

Synthetic oligonucleotide probes were synthesized based on the 100 base pair sequence discussed __supra__:

5'-TTC TGG CTG GGG AAC GAC CAC ATC CAC GCC CTG ACG GCC CAG GGA-3'
(sense)

5' GAA GTC CAC GAG GTC CAC CCG GAG CTC ATT GGT TCC CTG GGC CGT-3'
(antisense).

These probes were labelled with $[^{32}\gamma]P$ and were used to screen a cDNA library prepared from mRNA isolated from porcine uterus. The cDNA was inserted into λgt10 to form the library. The library was transferred onto nitrocellulose filters which were hybridized to the probes.

The filters were washed with 2xSSC, 0.1% SDS room temperature for 15 minutes, followed by 0.5xSSC, 0.1% SDS, 50°C for 20 minutes, and a cDNA clone was isolated. The insert was short, so this clone was used to rescreen the library, using slightly higher stringency washes from the first set of conditions (2xSSC, 0.1% SDS, room temperature 15 minutes, followed by 0.2xSSC, 0.1% SDS, 60°C, twenty minutes).

The isolated cDNA clone was sequenced, and thus is presented in SEQ ID NO: 5, attached hereto.

The foregoing experiments demonstrate the existence of several receptor like binding proteins for TGF-ß1. The term "receptor like" is used to distinguish these molecules generically from other molecules which have been referred to as "TGF-ß1 binding proteins". The previously described molecules are substances which are complexed to the TGF-ß1 molecule intracellularly and appear to be necessary to permit extracellular passage of the TGF-ß1. In contrast, there was no evidence of the molecules of this invention being complexed to TGF-ß1 when isolated. As such, they show "receptor like" properties in that they bind to and remove TGF-ß1 from solution, but "receptor" is generally used to refer to a membrane bound material which is involved in reception of the target molecule. There is no evidence to link the described and claimed molecules of the invention to such a role, thus they are referred to as "receptor-like" rather than receptors.

The data derived from the experiments reported herein supported the hypothesis that the TGF-ß1 binding proteins described herein are related as monomer, dimer and timer of the amino acid sequence described in e.g., SEQ ID NO: 5.

The molecules appear to be glycoproteins based upon their ability to bind to wheat germ agglutinin columns, and the size reduction of the 40 kd molecule following endoglycosidase treatment.

The three molecules do not appear to have proteoglycan structures and are, therefore, clearly distinct from type

III TGF-ß receptor, which is a proteoglycan and is referred to as "betaglycan". Additional comparison to, e.g., decorin (Yamaguchi et al., Nature 346: 281-284 (1990)); α2 macroglobulin (O'Connor-McCourt, et al., J. Biol. Chem. 262: 14090-14099 (1987)); and type IV collagen (Paralker et al., Dev. Biol. 143: 303-308 (1991)), are not warranted because all of these molecules have size and subunit compositions different from the molecules described herein, and are secreted molecules, unlike those described and claimed herein.

The sequence does, however, suggest a structure similar to that possessed by tenascin, as well as a collagen like domain.

The ability of these substantially pure receptor like TGF-ß1 binding glycoproteins to bind TGF-ß1 renders them useful in a number of ways. As indicated by the foregoing experiments, all three molecules bound to TGF-ß1 on a column. As such, each can be used as a "probe" to detect TGF-ß1 in a sample. Contact of the sample with the purified glycoprotein, followed by analysis for binding provides an assay method for TGF-ß1. In addition, the ability of the glycoproteins to bind TGF-ß1 makes them useful as therapeutic agents for preventing the binding of TGF-ß1 to a cell with an actual receptor, thereby inhibiting the effect of the TGF-ß1 if a sufficient amount of the glycoprotein is added. Other uses for the materials, such as an immunogen for production of antibodies, will be clear to the artisan and need not be set forth here.

Isolation of cDNA, as described herein, puts the artisan in possession of its complementary structure, as the complementary nature of DNA is well known. The deciphering of the amino acid sequence and the cDNA sequence will be seen to put the artisan in possession of the tools to isolate the genomic DNA sequence coding for the TGF-ß1 binding protein monomer. To the same end, conventional techniques of microbioloby may be used to

transfect cells, be they prokaryotic or eukaryotic, with the coding DNA (genomic or complementary). COS cells may be mentioned as one example of the type of cell which can be so transformed, but other cell types are readily accessible to the skilled artisan, and need not be discussed further.

Any cell receptive to transformation with the subject nucleic acid sequences will be seen to be capable of coding the 35-40 kd monomer. Those cells possessing the means to dimerize and trimerize the monomers will be capable of producing the d- and trimers discussed herein.

It is well known that nucleic acid sequences can be used as probes for those cells which express the binding protein. Such antisense sequences may also be used to bind to and inhibit the expression of the binding protein when this is appropriate. Other uses of these sequences will be clear to the skilled artisan.

The terms and expressions which have been employed are used as terms of description and not of limitation, and there is no intention in the use of such terms and expressions of excluding any equivalents of the features shown and described or portions thereof, it being recognized that various modifications are possible within the scope of the invention.

Claims

1. Isolated nucleic acid molecule which codes for a receptor like transforming growth factor ß1 binding protein molecule.

2. Isolated nucleic acid molecule complementary to the nucleic acid molecule of claim 1.

3. Isolated nucleic acid molecule of claim 1, wherein said molecule is cDNA.

4. Isolated nucleic acid molecule of claim 1, wherein said molecule if mRNA.

5. Isolated nucleic acid molecule of claim 1, wherein said molecule is genomic DNA.

6. Isolated nucleic acid molecule of claim 3, having the sequence set forth in SEQ ID NO: 5.

7. Isolated cell transfected with the nucleic acid molecule of claim 1.

8. The cell of claim 7, wherein said cell is a COS cell.

9. Substantially pure, receptor like transforming growth factor ß1 binding protein containing molecule, characterized by a molecular weight of from 35-40 kd as determined by SDS-PAGE.

10. Substantially pure, receptor like transforming growth factor ß1 binding protein containing molecule, characterized by a molecular weight of from 75-80 kd as determined by SDS-PAGE.

11. Substantially pure, receptor like transforming growth factor ß1 binding protein containing molecule, characterized by a molecular weight of 160 kd as determined by SDS-PAGE.

12. Method for identifying transforming growth factor ß1 binding protein in a sample, comprising contacting said sample to the substantially pure, receptor like binding protein containing molecule of claim 9 and determining binding to said binding glycoprotein as a determination of transforming growth factor 1 in said sample.

13. Method for identifying transforming growth factor ß1 binding protein in a sample, comprising contacting said sample to the substantially pure, receptor like binding protein containing molecule of claim 10 and determining binding to said binding protein containing molecule as a determination of transforming growth factor 1 in said sample.

14. Method for identifying transforming growth factor ß1 binding protein in a sample, comprising contacting said sample to the substantially pure, receptor like binding protein containing molecule of claim 11 and determining binding to said binding protein containing molecule as a determination of transforming growth factor ß1 in said sample.

15. Method for inhibiting the effect of transforming growth factor ß1 on a cell comprising administering an amount of the substantially pure, receptor like transforming growth factor ß1 binding protein containing molecule of claim 9 to a subject sufficient to inhibit said transforming growth factor ß1.

16. Method for inhibiting the effect of transforming growth factor ß1 on a cell comprising administering an

amount of the substantially pure, receptor like transforming growth factor ß1 binding protein containing molecule of claim 10 to a subject sufficient to inhibit said transforming growth factor ß1.

17. Method for inhibiting the effect of transforming growth factor ß1 on a cell comprising administering an amount of the substantially pure, receptor like transforming growth factor ß1 binding protein containing molecule of claim 11 to a subject sufficient to inhibit said transforming growth factor ß1.

18. Peptide fragment selected from the group consisting of:
(a) V(D)LV(D)FEGNHQFA
(b) VVGLEGSDKLSILR
(c) VFGSQLGE
(d) YLGGSHGSFA
(e) VVGLEGSDKLSILR
(f) CP*GLP*GAAGP
(g) DWAAY
and (h) RGFGSQLGEFWLGNDHIHALTAQGTNELXVDLVFEGNHQFA
where P* is hydroxyproline.

19. Isolated antibody which specifically binds to a receptor like transforming growth factor ß1 binding protein molecule.

20. The antibody of claim 19, wherein said antibody is a monoclonal antibody.

21. The antibody of claim 19 or 20, wherein said antibody binds to a receptor like transforming growth factor ß1

binding protein having molecular weight of from 35-40 kd as determined by SDS-PAGE.

22. The antibody of claim 19 or 20, wherein said antibody binds to a receptor like transforming growth factor ß1 binding protein having molecular weight of from 75-80 kd as determined by SDS-PAGE.

23. The antibody of claim 19 or 20, wherein said antibody binds to a receptor like transforming growth factor ß1 binding protein having molecular weight of from 160 kd as determined by SDS-PAGE.

AMENDED CLAIMS
[received by the International Bureau on 12 October 1992 (12.10.92) ;
original claims 1, 9-17 and 19-23 amended ;
remaining claims unchanged (4 pages)]

1. Isolated nucleic acid molecule which codes for a membrane derived, receptor like transforming growth factor ß1 binding protein molecule.

2. Isolated nucleic acid molecule complementary to the nucleic acid molecule of claim 1.

3. Isolated nucleic acid molecule of claim 1, wherein said molecule is cDNA.

4. Isolated nucleic acid molecule of claim 1, wherein said molecule if mRNA.

5. Isolated nucleic acid molecule of claim 1, wherein said molecule is genomic DNA.

6. Isolated nucleic acid molecule of claim 3, having the sequence set forth in SEQ ID NO: 5.

7. Isolated cell transfected with the nucleic acid molecule of claim 1.

8. The cell of claim 7, wherein said cell is a COS cell.

9. Substantially pure, membrane derived receptor like transforming growth factor ß1 binding protein containing molecule, characterized by a molecular weight of from 35-40 kd as determined by SDS-PAGE.

10. Substantially pure, membrane derived receptor like transforming growth factor ß1 binding protein containing molecule, characterized by a molecular weight of from 75-80 kd as determined by SDS-PAGE.

11. Substantially pure, membrane derived receptor like transforming growth factor ß1 binding protein containing molecule, characterized by a molecular weight of 160 kd as determined by SDS-PAGE.

12. Method for identifying transforming growth factor ß1 binding protein in a sample, comprising contacting said sample to the substantially pure, membrane derived receptor like binding protein containing molecule of claim 9 and determining binding to said binding glycoprotein as a determination of transforming growth factor 1 in said sample.

13. Method for identifying transforming growth factor ß1 binding protein in a sample, comprising contacting said sample to the substantially pure, membrane derived receptor like binding protein containing molecule of claim 10 and determining binding to said binding protein containing molecule as a determination of transforming growth factor 1 in said sample.

14. Method for identifying transforming growth factor ß1 binding protein in a sample, comprising contacting said sample to the substantially pure, membrane derived receptor like binding protein containing molecule of claim 11 and determining binding to said binding protein containing molecule as a determination of transforming growth factor ß1 in said sample.

15. Method for inhibiting the effect of transforming growth factor ß1 on a cell comprising administering an amount of the substantially pure, membrane derived receptor like transforming growth factor ß1 binding protein containing molecule of claim 9 to a subject sufficient to inhibit said transforming growth factor ß1.

16. Method for inhibiting the effect of transforming growth factor ß1 on a cell comprising administering an amount of the substantially pure, membrane derived receptor like transforming growth factor ß1 binding protein containing molecule of claim 10 to a subject sufficient to inhibit said transforming growth factor ß1.

17. Method for inhibiting the effect of transforming growth factor ß1 on a cell comprising administering an amount of the substantially pure, membrane derived receptor like transforming growth factor ß1 binding protein containing molecule of claim 11 to a subject sufficient to inhibit said transforming growth factor ß1.

18. Peptide fragment selected from the group consisting of:
(a) V(D)LV(D)FEGNHQFA
(b) VVGLEGSDKLSILR
(c) VFGSQLGE
(d) YLGGSHGSFA
(e) VVGLEGSDKLSILR
(f) CP*GLP*GAAGP
(g) DWAAY
~~and~~ (h) RGFGSQLGEFWLGNDHIHALTAQGTNELXVDLVFEGNHQFA
where P* is hydroxyproline.

19. Isolated antibody which specifically binds to a membrane derived receptor like transforming growth factor ß1 binding protein molecule.

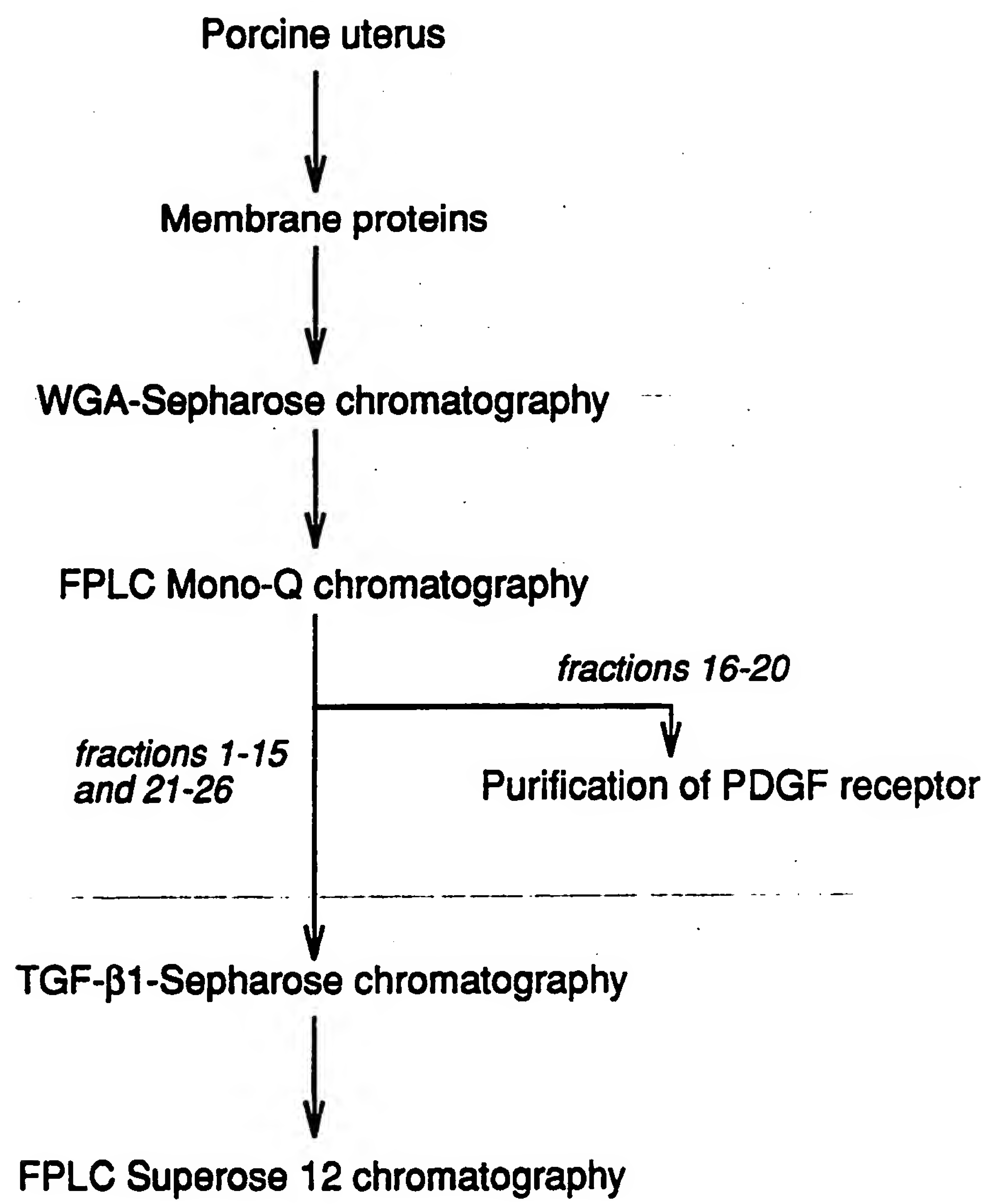
20. The antibody of claim 19, wherein said antibody is a monoclonal antibody.

21. The antibody of claim 19 or 20, wherein said antibody binds to a membrane derived receptor like transforming growth factor ß1 binding protein having molecular weight of from 35-40 kd as determined by SDS-PAGE.

22. The antibody of claim 19 or 20, wherein said antibody binds to a membrane derived receptor like transforming growth factor ß1 binding protein having molecular weight of from 75-80 kd as determined by SDS-PAGE.

23. The antibody of claim 19 or 20, wherein said antibody binds to a membrane derived receptor like transforming growth factor ß1 binding protein having molecular weight of from 160 kd as determined by SDS-PAGE.

FIG. 1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Membrane proteins
WGA-Sepharose chromatography
FPLC Mono-Q chromatography
fractions 16-20
fractions 1-15
and 21-26
Purification of PDGF receptor
TGF-β1-Sepharose chromatography
FPLC Superose 12 chromatography

FIG. 2

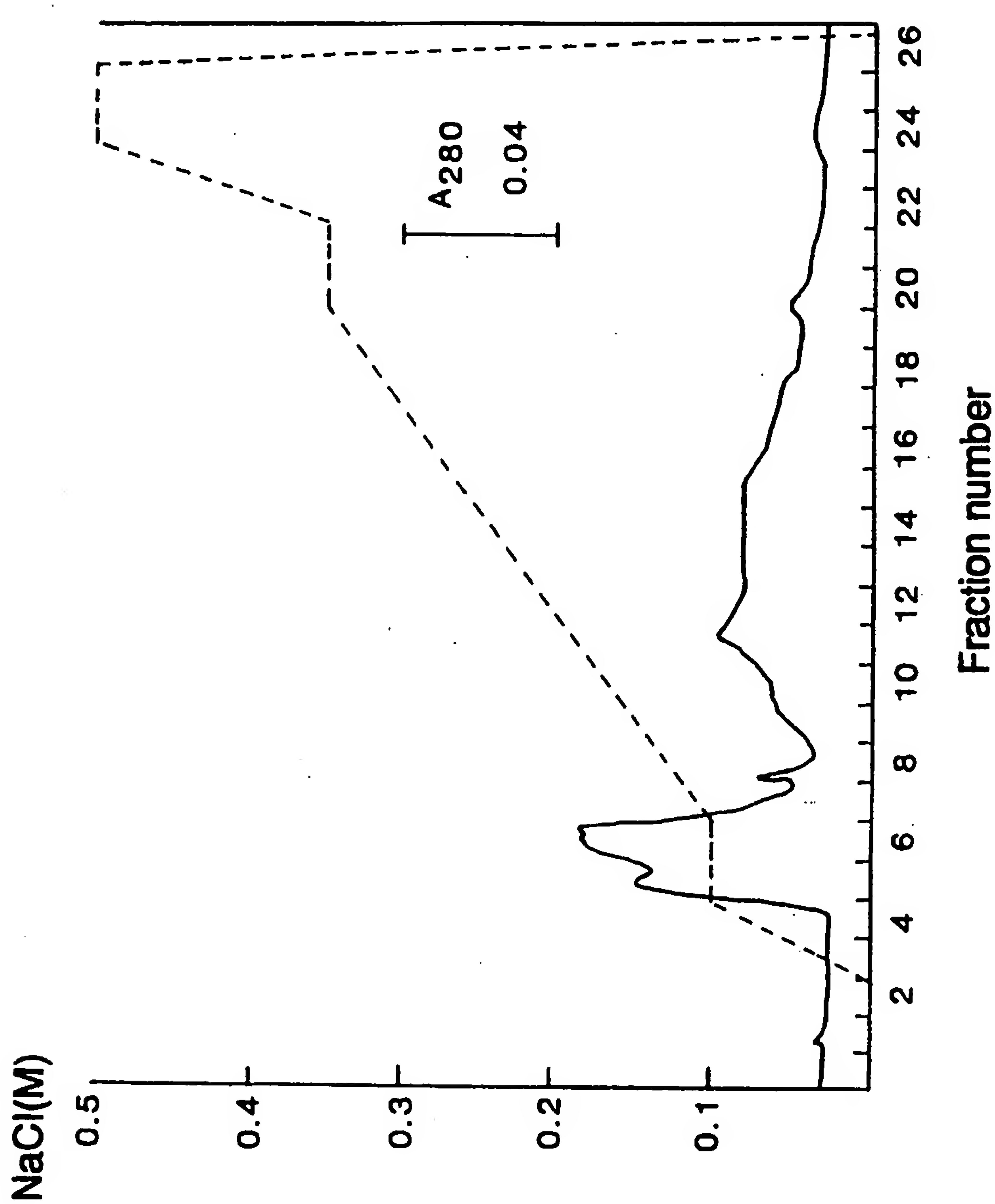
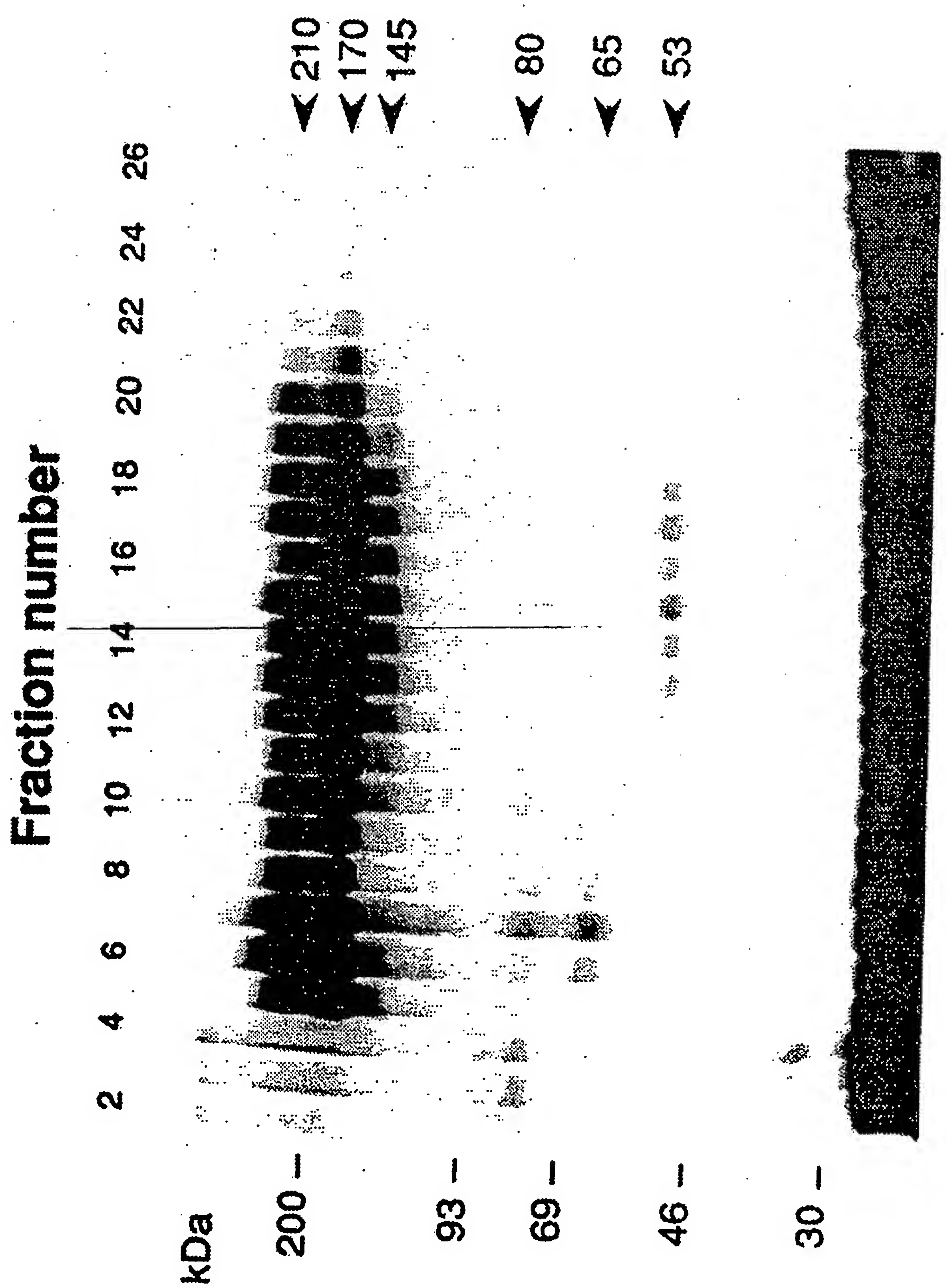

NaCl(M)
0.5
0.4
0.3
0.2
0.1
A_{280}
0.04
2
4
6
8
10
12
14
16
18
20
22
24
26
Fraction number

FIG. 3


Fraction number
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kDa
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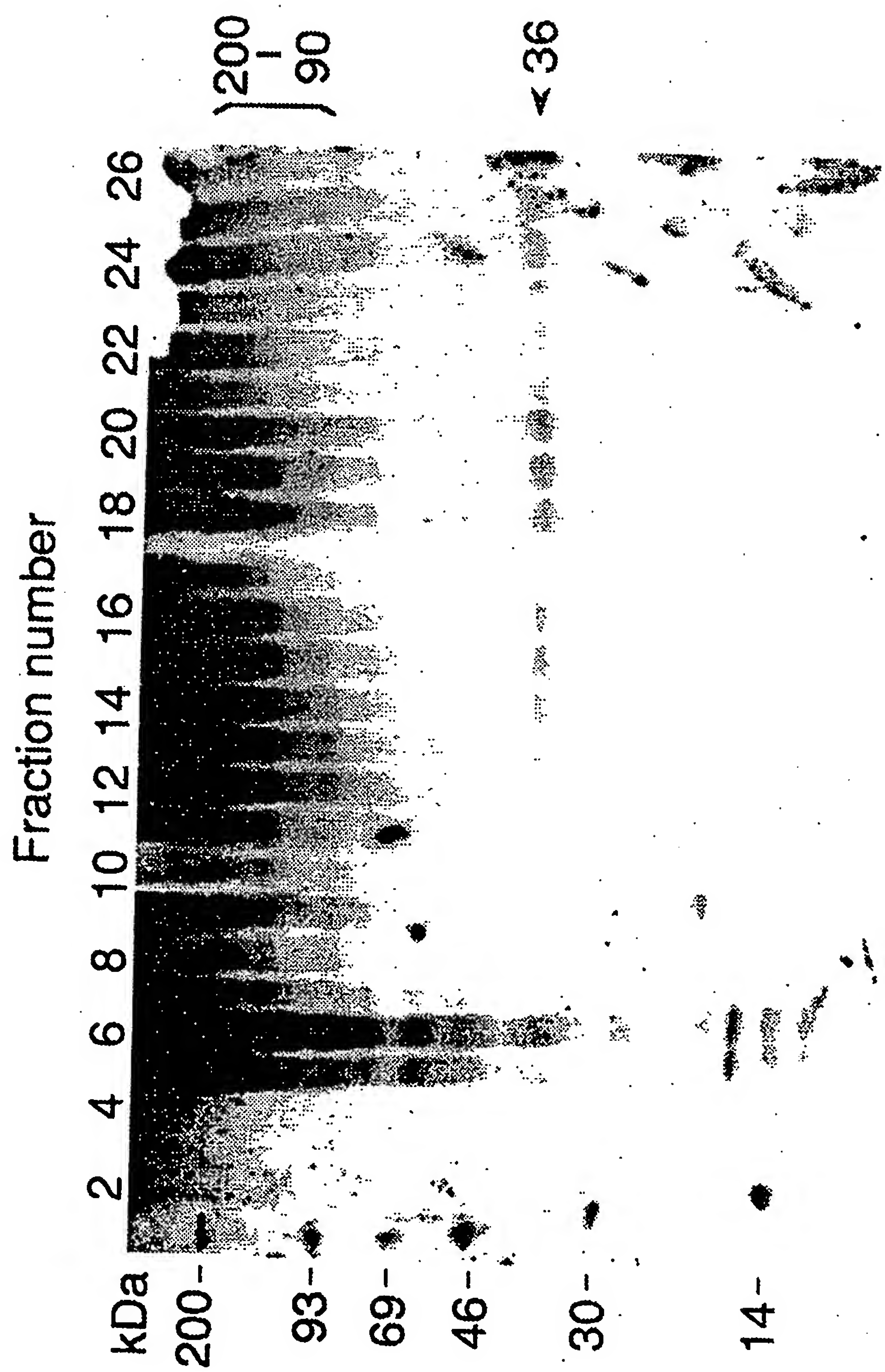
93
69
46
30
210
170
145
80
65
53

FIG. 4


Fraction number
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kDa
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69 -
46 -
30 -
14 -
200 - 90
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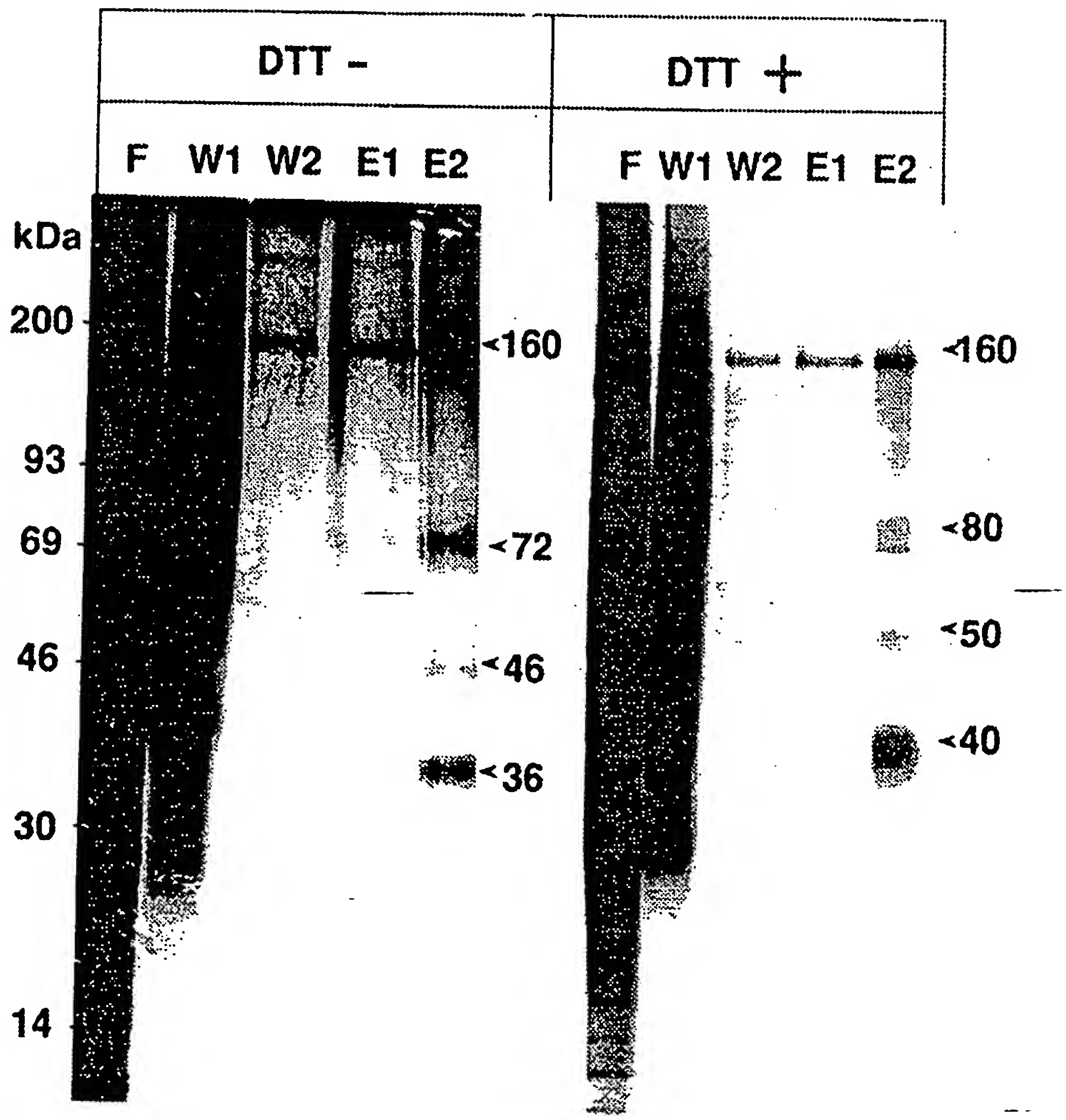
FIG. 5


DTT -
DTT +
F W1 W2 E1 E2
F W1 W2 E1 E2
kDa
200
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46
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<46
<36
<160
<80
<50
<40

FIG. 6

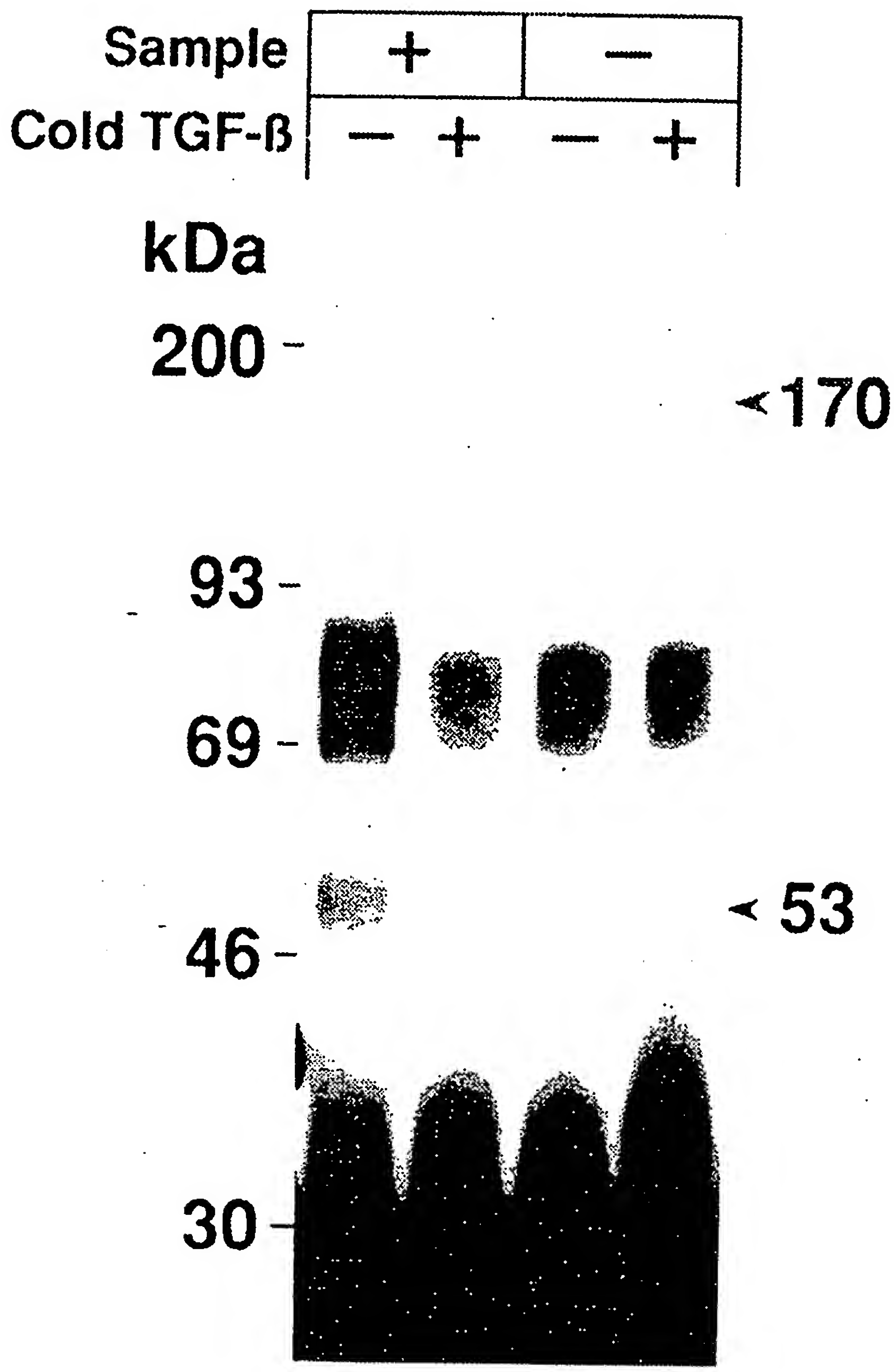


FIG. 7

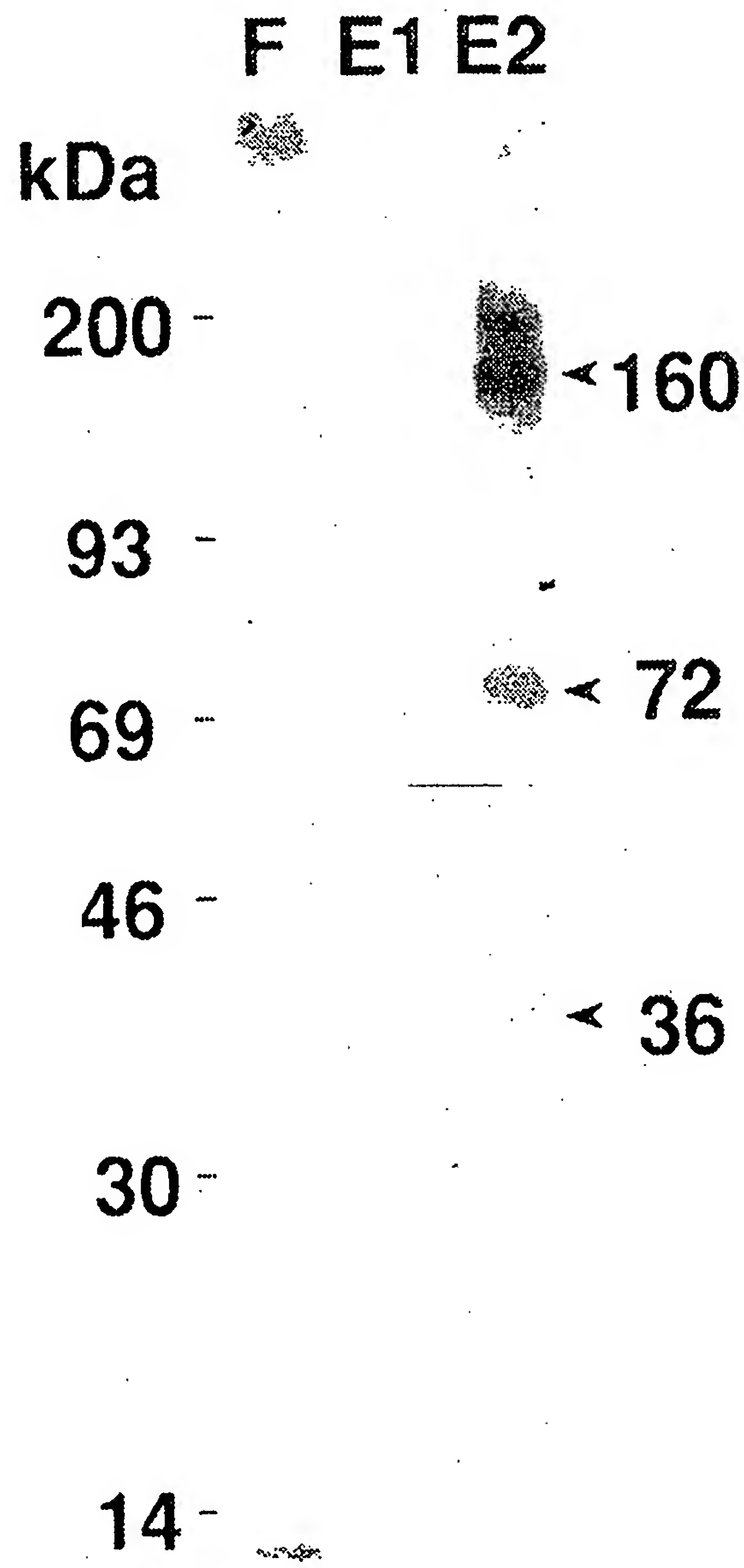


FIG. 8

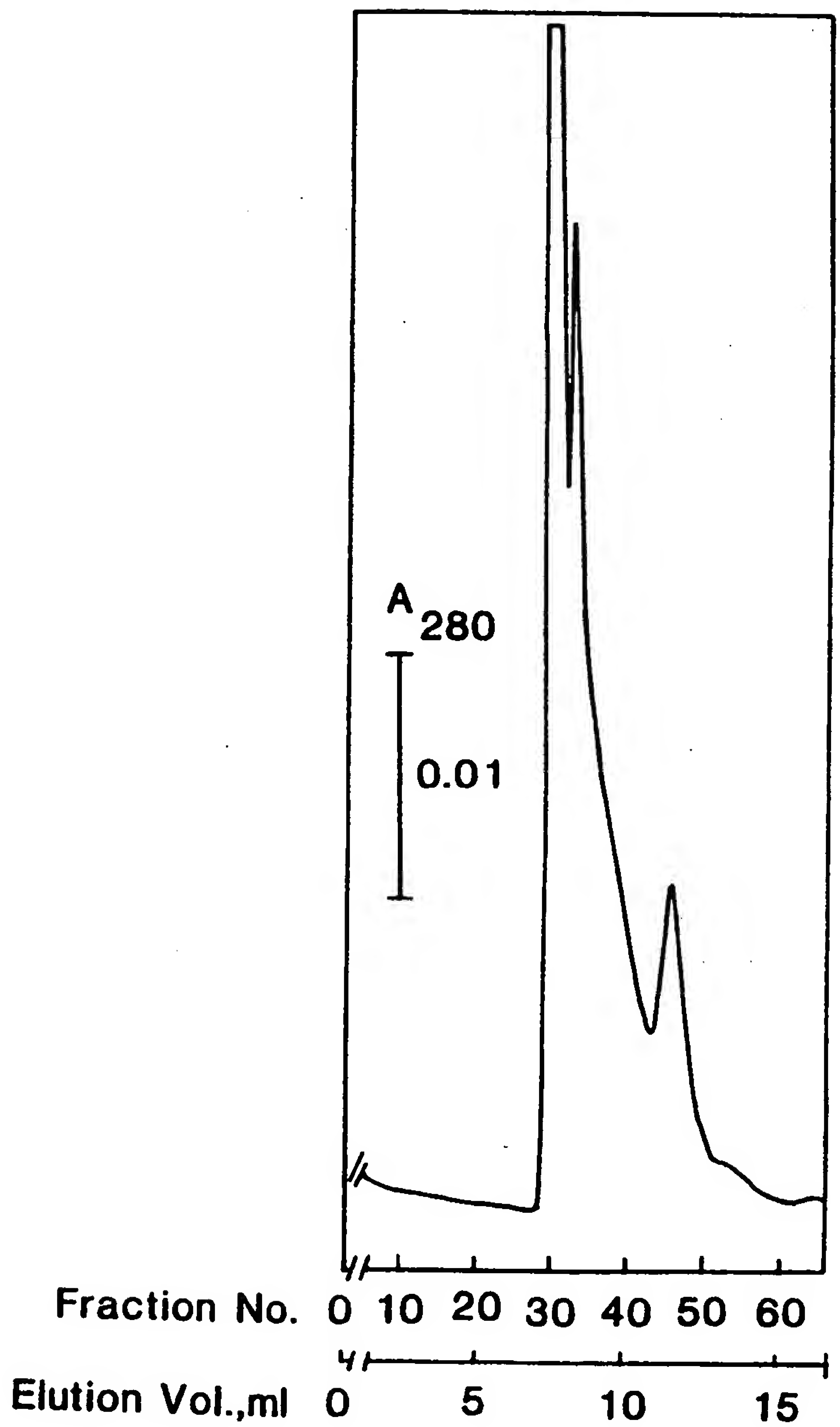
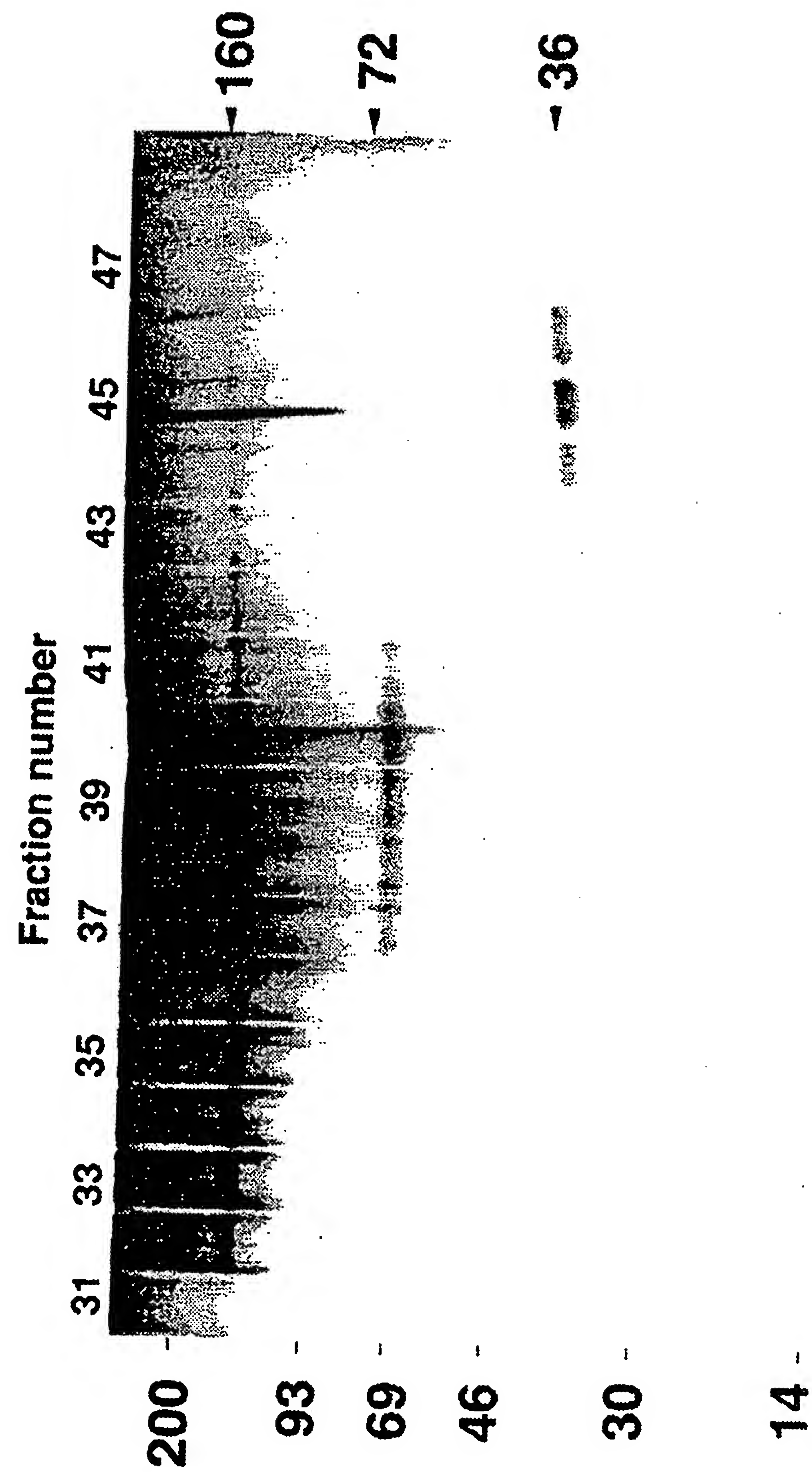


FIG. 9


Fraction number
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37
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41
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72
36
200
93
69
46
30
14

FIG. 10

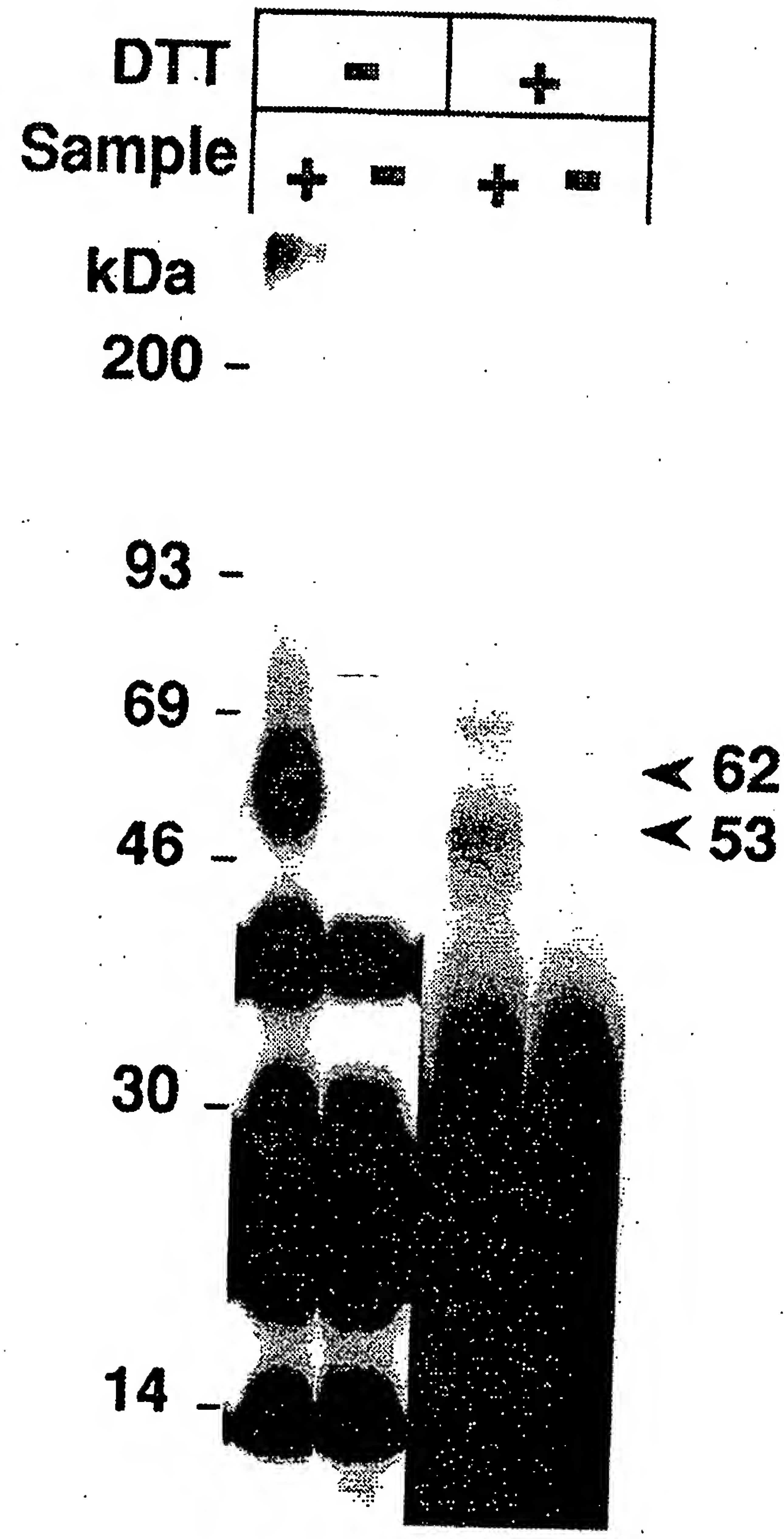

FIG. 11

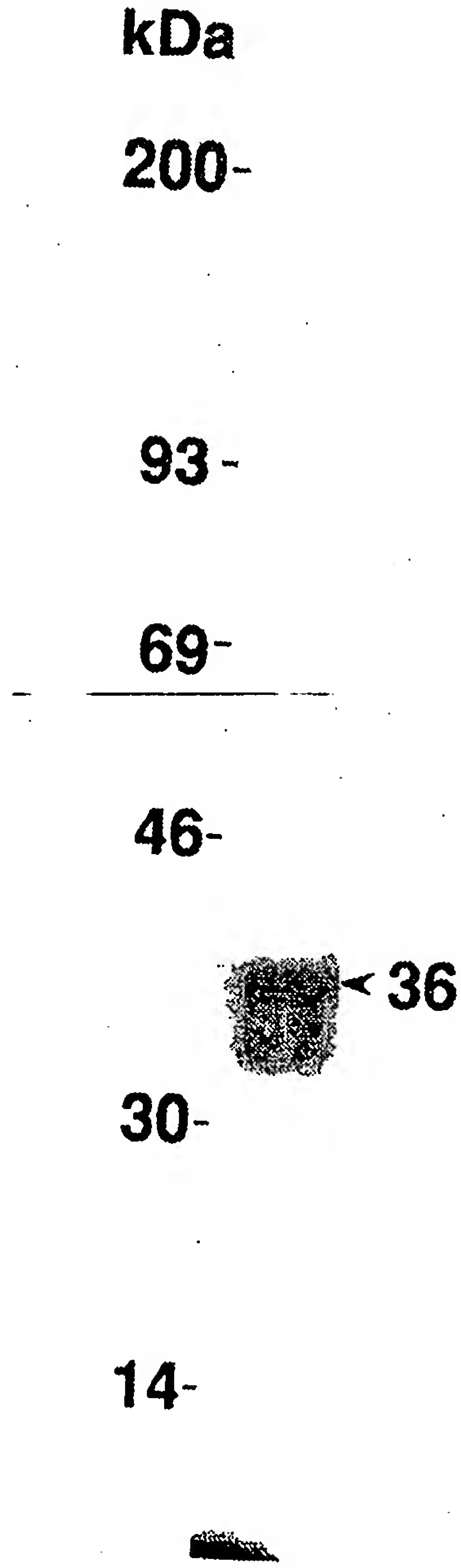

FIG. 12

```
GCGATGGACACACGCGGAGTGGCCGCGGCCATGAGGCCCCTGGTCCTGCTCGTTGCCTTC   60
A  M  D  T  R  G  V  A  A  A  M  R  P  L  V  L  L  V  A  F
CTGTGCACCGCAGCCCCAGCCCTCGACACCTGTCCAGAGGTCAAGGTGGTGGGTCTGGAG  120
 L  C  T  A  A  P  A  L  D  T  C  P  E  V  K  V  V  G  L  E
GGCTCGGACAAGCTCTCCATCCTCCGAGGCTGCCCGGGGCTGCCTGGAGCCGCAGGGCCC  180
 G  S  D  K  L  S  I  L  R  G  C  P  G  L  P  G  A  A  G  P
AAGGGAGAGGCGGGCGCCAGTGGACCGAAGGGAGGACAAGGCCCTCCCGGAGCCCCTGGG  240
 K  G  E  A  G  A  S  G  P  K  G  G  Q  G  P  P  G  A  P  G
GAGCCAGGACCCCCCGGGCCCAAAGGAGACCGAGGGGAGAAGGGCGAGCCTGGACCAAAA  300
 E  P  G  P  P  G  P  K  G  D  R  G  E  K  G  E  P  G  P  K
GGAGAGTCTTGGGAAACCGAGCAGTGTCTCACAGGACCTCGGACCTGCAAGGAGCTGCTG  360
 G  E  S  W  E  T  E  Q  C  L  T  G  P  R  T  C  K  E  L  L
ACCAGGGGGCACATTCTGAGCGGCTGGCACACCATCTACCTGCCAGACTGCCAGCCCCTG  420
 T  R  G  H  I  L  S  G  W  H  T  I  Y  L  P  D  C  Q  P  L
ACGGTGCTGTGTGACATGGACACGGATGGCGGGGGGTGGACCGTTTTCCAGCGCAGGAGC  480
 T  V  L  C  D  M  D  T  D  G  G  G  W  T  V  F  Q  R  R  S
GACGGGTCGGTGGACTTCTACCGGGACTGGGCCGCGTACAAGCGGGGCTTCGGCAGTCAG  540
 D  G  S  V  D  F  Y  R  D  W  A  A  Y  K  R  G  F  G  S  Q
CTGGGAGAGTTCTGGCTGGGGAACGACCACATCCACGCCCTGACGGCCCAGGGAACCAAT  600
 L  G  E  F  W  L  G  N  D  H  I  H  A  L  T  A  Q  G  T  N
GAGCTCCGGGTGGACCTCGTGGACTTCGAGGGCAACCACCAGTTTGCCAAGTACAGGTCC  660
 E  L  R  V  D  L  V  D  F  E  G  N  H  Q  F  A  K  Y  R  S
TTCCAGGTGGCAGACGAGGCAGAGAAGTACATGCTGGTCCTGGGAGCCTTTGTAGAGGGC  720
 F  Q  V  A  D  E  A  E  K  Y  M  L  V  L  G  A  F  V  E  G
AATGCAGGTGATTCCCTGACGTCCCACAACAACAGCCTGTTCACCACCAAAGACCAGGAC  780
 N  A  G  D  S  L  T  S  H  N  N  S  L  F  T  T  K  D  Q  D
AACGACCAGTACGCCTCAAATTGTGCAGTGCTGTACCAGGGAGCCTGGTGGTACAACAGC  840
 N  D  Q  Y  A  S  N  C  A  V  L  Y  Q  G  A  W  W  Y  N  S
TGTCACGTGTCCAACCTGAACGGCCGCTACCTCGGGGGCTCGCACGGGAGCTTTGCAAAC  900
 C  H  V  S  N  L  N  G  R  Y  L  G  G  S  H  G  S  F  A  N
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 G  V  N  W  S  S  G  K  G  Y  N  Y  S  Y  K  V  S  E  M  K
TTTCGGGCCACCTAGGGCGGGACAGTGCTTCCAGAACCCTCCCTGGGGAGGGGCCACGGG 1020
 F  R  A  T

GCTCCCGCTCACTATCCGCCCGGGTGTGAAGGGCCACATCCCAACCCTGGGGGGCGGCCA 1080

TGCCCTCTGCACCTCCACCAGCTTCCAATCTTCTGTCCCTCTCAGGAGGACAAGAGTGAC 1140

CGTTACTCCAGCAACATGTATTCTCAATAAAGACACTTGCTTACCCAAAAAAAAAA 1196
```

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US92/05199

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :A61K 37/02; C07K 3/02, 15/06, 15/28; C12N 15/00; G01N 33/567
US CL :Please See Extra Sheet.
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 436/503, 504; 514/12, 21; 530/324, 327, 328, 331, 350, 351, 395, 388.2, 389.1; 935/4, 11, 70

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, DIALOG,
search terms: TGF, beta, receptor, membrane

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	J. Darnell et al. "Molecular Cell Biology", published 1986 by Scientific American Books, Inc. (NY), pages 221, 222, 260-262. See entire document.	1-8
Y	Nature, Vol. 256, issued 07 August 1975, G. Kohler et al. "Continuous Cultures Of Fused Cells Secreting Antibody Of Predefined Specificity", pages 495-497, especially pages 495, column 1, and page 497, column 1.	19-23
X Y	The Journal Of Biological Chemistry, Volume 261, No. 1, issued 25 July 1986, S. Cheifetz et al., "Cellular Distribution Of Type I and Type II Receptors For Transforming Growth Factor-β", pages 9972-9978, especially the Abstract.	10 1-8,13, 18-20,22
X Y	Cell, Vol. 48, issued 13 February 1987, S. Cheifetz et al., "The Transforming Growth Factor-β System, A Complex Pattern Of Cross-Reactive Ligands And Receptors", pages 409-415, especially the Abstract and Figure 5.	10 1-8, 13, 18-20, 22

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be part of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search 01 September 1992	Date of mailing of the international search report 14 SEP 1992
Name and mailing address of the ISA/ Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. NOT APPLICABLE	Authorized officer JEFFREY E. RUSSEL Telephone No. (703) 308-0196

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	The Journal Of Biological Chemistry, Vol. 260, No. 5, issued 10 March 1985, J. Massague et al., "Cellular Receptors For Type β Transforming Growth Factor", pages 2636-2645, especially the Abstract.	10 1-8, 13, 18-20, 22
X Y	Nature, Vol. 338, No. 6211, issued 09 March 1989, K. Miyazono et al., "Role For Carbohydrate Structures In TGF-β1 Latency", pages 158-160, especially page 158, column 1.	1-3, 9 4-8, 12, 18-21
X Y	JP, A, 63-150300 (Toa Nenryo Kogyo KK) 22 June 1988, see the Abstract.	9, 15 1-8, 12, 18-21
X Y	The Journal Of Biological Chemistry, Vol. 263, No. 16, issued 05 June 1988, L. Wakefield et al., "Latent Transforming Growth Factor-β From Human Platelets", pages 7646-7654, especially the Abstract.	10 1-8, 13, 18-20, 22
X Y	Cell, Vol. 61, issued 15 June 1990, T. Kanzaki et al., "TGF-β1 Binding Protein: A Component Of The Large Latent Complex Of TGF-β1 With Multiple Repeat Sequences", pages 1051-1061, especially the Summary, pages 1053, column 2, last paragraph, and Figure 4	1-3, 7, 8, 11 4-6, 14, 18-20, 23

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US92/05199

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

436/503, 504; 514/12, 21; 530/324, 327, 328, 331, 350, 388.2, 389.1; 935/4, 11, 70